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Virus Particle Content of Smallpox Vaccines

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Calf lymph smallpox vaccines contain too much extraneous debris for an accurate assessment of their virus particle content. The process of partial purification of the vaccine utilizing enzymatic digestion by chymotrypsin, subtilisin, and collagenase solubilized enough debris to permit electron microscopic virus particle count. Enzyme treatment did not degrade or destroy the virus nor did it reduce the infective titer. Commercial vaccines studied ranged in virus content from 1.89×10^9 to 1.09×10^{11} virus particles/ml. The pocking efficiencies on the chorioallantoic membrane of some of these vaccines varied from 200 to 1,200 virus particles per pock-forming unit.

Measurements of smallpox vaccine potency are based not on antigenic virus particle content but on titrations of infectivity for different host systems, such as the production of a confluent lesion of a specified size on the skin of a rabbit (6), the pock titer on the chorioallantoic membrane (CAM) of embryonated eggs (11, 12), or the plaque titer in cell monolayers (7). Infectivity titers of the same vaccine sample may vary widely in these different hosts. The physical measurement of virus particle count provides a constant reference point in correlating biological titers. The ratio between count and titer is an indication of the efficiency of infectivity in that host system and serves as a valuable marker in characterizing and differentiating virus strains.

The virus particle content of calf lymph smallpox vaccines is difficult to establish because of the extraneous debris that is a by-product of its manufacture. An indirect method of assessing virus content involving a series of extractions of virus was developed by Sharp (10). This method depends on mathematical relationships which may not always be possible to attain experimentally. Our aim was to devise a simple method of partial purification of vaccine which would permit a direct electron microscopic count of virus.

MATERIALS AND METHODS

Types of vaccine. Glycerinated (wet) and lyophilized (dried) smallpox vaccines derived from calf lymph were produced by United States licensed manufacturers. The U.S. Reference Smallpox Vaccine, lot 2, was obtained from Division of Biologics Standards, National Institutes of Health.

Cell cultures. BS-C-1 and RK-13 lines were obtained from the Tissue Culture Section of the Division of Biologics Standards.

Enzymes. Enzymes were purchased from Lederle Laboratories, Sigma Chemical Co., Worthington Biochemical Corp., and Calbiochem. Subtilisin, α-chymotrypsin, snake venom (from *Crotalus adamanteus*), and collagenase were used at a concentration of 100 μg/ml of vaccine diluted 1:10.

Particle counts. Virus particle counts were made by the agar sedimentation method of Sharp (9).

Titrations. Plaque titrations were performed in cell cultures by the agar overlay method of Galasso and Sharp (4). Vaccine titrations on the CAM of 12-day-old embryonated chicken eggs were performed by the Laboratory of Control Activities, Division of Biologics Standards as described in reference 3.

Sonic treatment. Vaccine samples were sonic-treated in a Raytheon sonic oscillator (10 kv) for 1 min, at maximum intensity.

RESULTS

Electron microscopic examination of smallpox vaccine. Calf lymph smallpox vaccine contains a large amount of particulate debris, rendering an accurate assessment of virus content impossible in untreated material (Fig. 1A). Attempts to reduce this debris with the use of a number of enzymes (trypsin, streptokinase, streptodornase, ribonuclease, deoxyribonuclease, and Pronase) were unsuccessful. If the enzyme treatment was effective enough to break up the debris, a mass of small "dustlike" particles resulted which obscured the virus in the microscopic field. Some of these enzymes markedly degraded the virus, making recognition difficult.

Partial purification of smallpox vaccine. An adaptation of a method for purifying adenovirus devised by Burnett et al. (1) was successful in eliminating sufficient debris to permit acceptable virus particle counts. Snake venom, α-chymotrypsin, and subtilisin were incubated with a 1:10

dilution of the vaccine. This treatment produced virus preparations of a purity illustrated in Fig. 1B. Experimentation with different combinations of these enzymes revealed that snake venom had little effect on the preparation and that a combination of α-chymotrypsin and subtilisin was sufficient for good results.

However, many vaccines contain a considerable

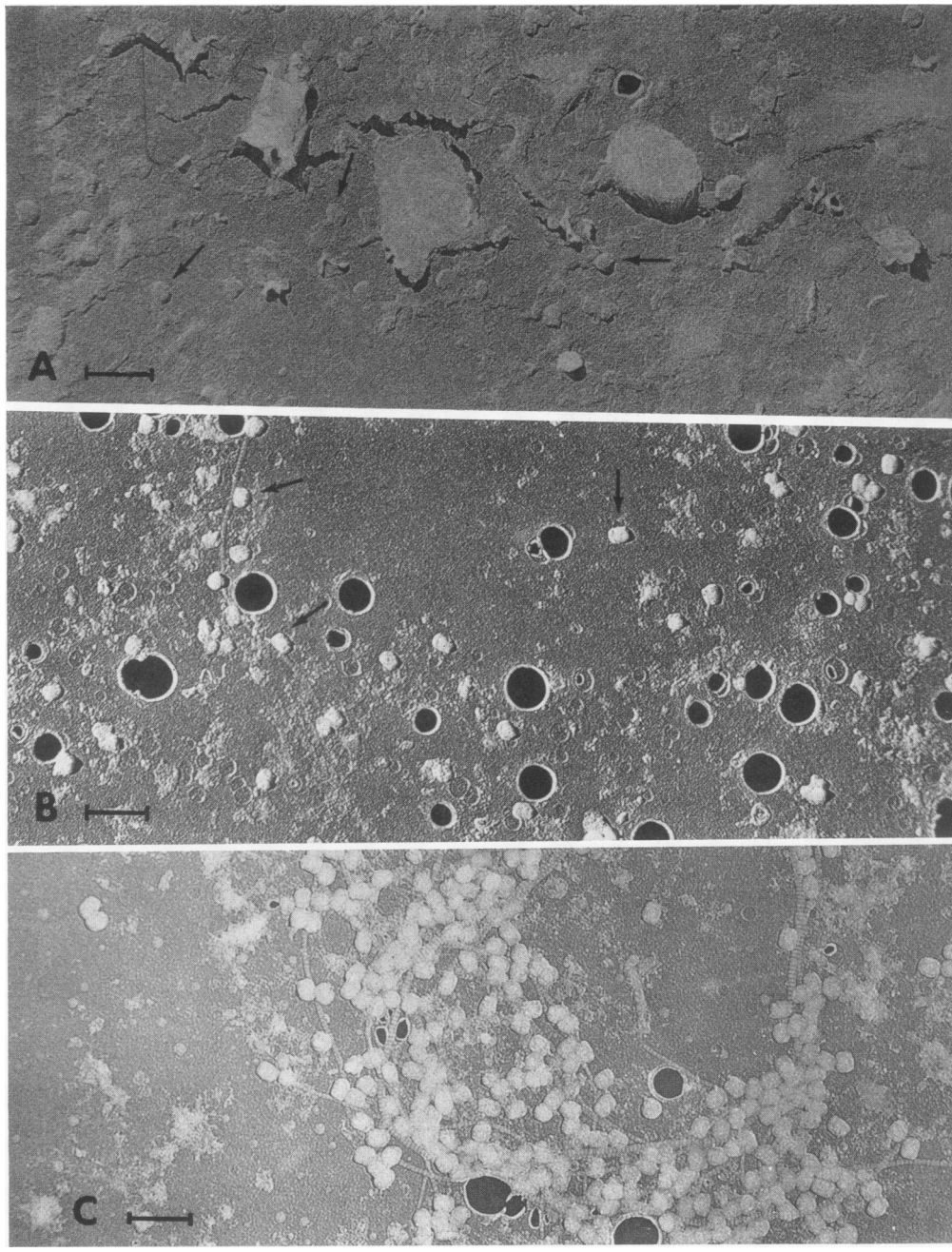

FIG. 1. *Electron micrographs of smallpox vaccines. (A) Untreated vaccine diluted 1:2,000; (B) vaccine incubated with α-chymotrypsin, subtilisin, and snake venom and diluted 1:2,600; (C) tangle of collagen fibers with vaccinia particles attached, from partially purified smallpox vaccine, diluted 1:2,600. Bar represents 1 μm. Arrows indicate virus particles. The preparations are chromium-shadowed.*

amount of collagen fibers (Fig. 1C) to which virus seems to adhere. Masses of this type upset the distribution of virus particles desirable for accurate counting. We added treatment with collagenase plus Ca^{2+} to the procedure and found that, although collagen fibers still persisted, very few virus particles remained attached to them.

The standard procedure we have adopted for enzyme treatment of vaccine for particle counting is as follows. (i) The vaccine is diluted 1:10 with phosphate-buffered saline (PBS; *p*H 7.2) plus 5 mg per 100 ml of calcium ion (since collagenase has an absolute requirement for calcium) and sonic-treated for 1 min. (ii) α-Chymotrypsin and subtilisin are added at a concentration of 100 μg each per ml of diluted vaccine. The mixtures are incubated at 37 C for 30 min. (iii) Collagenase is added at a concentration of 100 μg per ml of diluted vaccine, and incubation at 37 C is continued for 45 min. (iv) The mixtures are sonic-treated for 1 min and then diluted in PBS to a final concentration of 1:2,600 of the original vaccine sample. (v) The virus is sedimented on agar in the Sharp-Sorvall counting rotor at a speed of 10,000 rev/min for 15 min and prepared for electron microscopic count.

Particle counts on commercial glycerinated vaccines enzyme-treated in this manner are listed in Table 1. Virus content of these vaccines ranged from 2.4×10^{10} to 10.9×10^{10} virus particles (VP)/ml.

TABLE 1. *Particle counts on enzyme-treated vaccines*

Vaccine	No. of counts	Mean particle count (per ml)	Standard deviation
1	3	2.4×10^{10}	0.92×10^{10}
2	4	10.9×10^{10}	2.80×10^{10}
3	7	6.2×10^{10}	0.80×10^{10}
4	6	7.5×10^{10}	1.77×10^{10}
5	3	5.3×10^{10}	1.78×10^{10}

Effect of enzyme treatment on infectivity. Duplicate plaque titrations of untreated and enzyme-treated vaccine were made in tissue culture cells. Two cell lines were used, BS-C-1 and RK-13 cells, and three glycerinated vaccines were tested in each cell line. The titers are recorded in Table 2 with the amount of change in titer after enzyme treatment. In most cases, there was a slight enhancement of titer after enzyme treatment.

Correlation of count and plaque titer in untreated and enzyme-treated reference vaccine. The DBS reference smallpox vaccine is sufficiently free from debris to permit a reasonably accurate direct count of virus particles with no treatment other than sonic treatment. This material was counted with and without enzyme treatment (Table 3). There is essentially no difference in the mean counts of the vaccine with and without enzyme treatment. The greater variability of the counts on the untreated vaccine is due to the presence of some debris, making virus recognition more difficult than in the enzyme-treated specimens.

The infectivity of counted preparations of untreated and enzyme-treated reference vaccine was determined in RK-13 cells (Table 4). The plaquing efficiency of these two preparations was essentially the same.

Pocking efficiency of vaccines. We performed particle counts on six commercial vaccines which had previously been tested for potency on the CAM by the Laboratory of Control Activities, Division of Biologics Standards, under their carefully controlled conditions (3). These results are shown in Table 5. Vaccines A through E were lyophilized vaccines; F was glycerinated. Vaccines A through C were free enough from debris that counts could be made on the untreated material. Vaccines D through F required enzyme treatment to reduce the debris sufficiently to per-

TABLE 2. *Titrations of untreated and enzyme-treated vaccines*

Vaccine	BS-C-1 titer[a]	Δ[b]	BS-C-1 titer	Δ	RK-13 titer	Δ	RK-13 titer	Δ
I								
Untreated	7.58		7.32		7.74		8.04	
Enzyme-treated	7.77	+0.19	7.38	+0.06	8.03	+0.29	8.36	+0.32
II								
Untreated	7.45		6.99		7.53		7.79	
Enzyme-treated	7.86	+0.41	7.56	+0.57	8.15	+0.62	8.56	+0.77
III								
Untreated			6.89		7.62		7.79	
Enzyme-treated			6.26	−0.63	7.20	−0.42	7.94	+0.15

[a] Log_{10} PFU/ml.
[b] Change in titer after enzyme treatment (log_{10}).

TABLE 3. *Particle counts on DBS reference vaccine*[a]

Expt	Untreated	Enzyme-treated
1	10.69	10.60
2	10.82	10.67
3	10.51	10.65
4	10.74	10.68
Mean	10.70	10.65

[a] Expressed as $\log_{10}$ VP/ml.

TABLE 4. *Infectivity of untreated and enzyme-treated reference vaccine in RK-13 cells*

Determination	Untreated	Enzyme-treated
Titer (PFU/ml)	3.7×10^7	2.5×10^7
Count (VP/ml)	5.46×10^{10}	4.77×10^{10}
Particles/PFU	1,480	1,908

TABLE 5. *Pocking efficiency of vaccines*[a]

Vaccine	Particle count (VP/ml)	CAM titer (PKFU/ml)	Pocking efficiency (VP/PKFU)
A	1.89×10^9	4.0×10^6	470
B	2.96×10^9	2.5×10^6	1,180
C	3.40×10^9	4.0×10^6	850
D	2.64×10^{10}	6.31×10^7	418
E	4.33×10^{10}	1.00×10^8	433
F	1.34×10^{10}	7.12×10^7	188

[a] Vaccines A through E were lyophilized vaccines; F was glycerinated. A through C were counted without enzyme treatment; D through F required enzyme treatment.

mit particle counts. Pocking efficiency of vaccines tested ranged from approximately 200 VP/pock-forming unit (PKFU) for the glycerinated vaccine to about 1200 VP/PKFU for one of the lyophilized vaccines.

DISCUSSION

Enzymatic digestion of calf lymph smallpox vaccine with chymotrypsin, subtilisin, and collagenase reduces the extraneous debris to a point where an accurate quantitative assessment of virus content can be made. The enzyme treatment does not destroy the virus nor reduce its infectivity. Support for this claim is provided by the following evidence. (i) Particle counts performed on the Division of Biologics Standards reference vaccine before and after enzyme treatment show no significant difference in virus content. (ii) Plaque titrations of commercial vaccine with and without enzyme treatment are essentially similar with a slight enhancement of titer generally noted on the enzyme-treated samples. An enhancement of plaque titer after treatment with proteolytic enzymes was noted by Kim et al. (5) and Klapper and Gifford (Bacteriol. Proc., p. 183, 1968). Although this increase in titer could be due to factors such as dispersal of aggregates or partial uncoating of the virions, the fact that there is an enhancement rather than a decrease in titer lends weight to the contention that virus is not destroyed by the enzymatic action. (iii) Titrations of the reference vaccine with which particle counts could be obtained on both the untreated and enzyme-treated samples showed close agreement on the particles to plaque-forming units (PFU) ratio.

Commercial vaccines used in this study ranged in virus particle content from 1.89×10^9 to 1.09×10^{11} VP/ml. The three vaccines studied which had particle counts of the order of magnitude of 10^9 VP/ml (Table 5; vaccines A through C) were intended for jet gun administration. Vaccines given by this method are diluted to show a potency by CAM titer 1.5 logs lower than vaccines to be administered by the scarification method, since jet gun inoculation is more efficient than the multiple pressure inoculation system (8). Most of the vaccines used in this study contained between 10^{10} and 10^{11} VP/ml and were intended for multiple pressure injection. Comparison of the particle count with infectivity titers on the CAM (Table 5) revealed as much as a sixfold difference in pocking efficiencies among the six vaccines investigated. All of these vaccines met the Division of Biologics Standards potency requirements, but vaccine F was two to six times as efficient in the CAM test system as the other five vaccines.

The particle-infectivity ratio in a given host system is a valuable strain marker, which may prove useful in the search for attenuated smallpox vaccine strains. It was previously reported (2) that the WR strain of vaccinia passed 324 times in L cells had a plaquing efficiency of 10 VP/PFU but had lost its ability to cause a lesion in rabbit skin, whereas the early passage WR (passed 21 times in L cells) plaqued with an efficiency of 200 VP/PFU, and as little as 1 PFU could cause a lesion in a rabbit. The process of adaptation to efficient growth in one host system may result in lessened virulence in another host. The improved particle-infectivity ratio is evidence of adaptation.

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